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Knockdown of *TP53* in *ASXL1* negative background rescues apoptotic phenotype of human hematopoietic stem and progenitor cells but without overt malignant transformation

With increasing age, the incidence of haematological malignancies rises, including myelodysplastic syndrome (MDS). MDS is characterized by ineffective hematopoiesis, a defect of differentiation in one or more hematopoietic lineages, which can progress to acute myeloid leukemia (AML) in time.¹ Throughout the development of MDS and AML, a vast subset of different driver mutations can be identified that are prognostic for disease outcome. In low-risk MDS, frequently one driver mutation can be recognized, which is accompanied by a second mutation in the context of disease progression.¹ Two genes that are found to be co-mutated in MDS patients are *ASXL1* and *TP53*.² In patients, *TP53* mutations are often observed in complex karyotypes and predict unfavourable prognosis.² Functional knockout stud-

ies of the mouse *TP53* gene, *Trp53*, revealed resistance to apoptosis after induced DNA damage.³ Mutations in *ASXL1* are also generally associated with an unfavourable prognosis.⁴ Loss of function of the epigenetic modifier *ASXL1* in mice has been found to be embryonic lethal and, after long latency, *ASXL1* heterozygous mice developed MDS-like phenotypes.⁵ Additionally, *ASXL1* loss leads to reduced numbers of stem and progenitors *in vitro* and *in vivo*, accompanied by increased apoptosis and decreased H3K27me3 levels.^{6,7} Nevertheless, malignant transformation has only been observed in cells with altered genetic background, suggesting that *ASXL1* mutations need to be accompanied by additional mutations.⁸

The aim of this study was to address whether simultaneous loss of *ASXL1* and *TP53* can rescue the adverse phenotype in human hematopoietic stem and progenitor cells (HSPC) upon *ASXL1* knockdown and promote transformation.

To delineate the role of *ASXL1* and *TP53* loss in hematopoiesis, we used an RNAi approach. We trans-

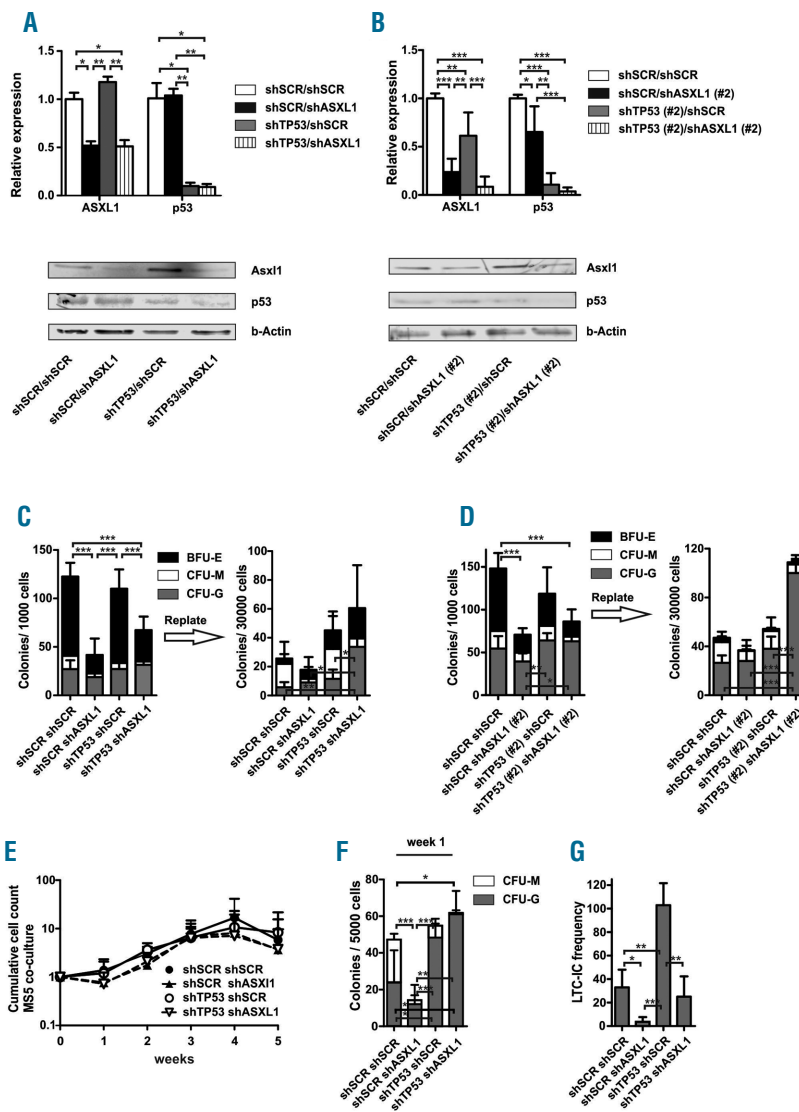


Figure 1. Double loss of *ASXL1* and *TP53* rescues replating potential of colonies. (A) Gene expression hairpin #1 normalized to NACA and RPS11 (shown are internal repeats) and protein levels. (B) Gene expression hairpin #2 normalized to NACA and RPS11 and protein levels. (C) CFC analysis of CB cells expressing hairpin #1 shSCR/shSCR, shSCR/shASXL1, shTP53/shSCR, shTP53/shASXL1 (N=4). (D) CFC analysis of CB cells expressing hairpin #2 shSCR/shSCR, shSCR/shASXL1, shTP53/shSCR, shTP53/shASXL1 (N=3). (E) Cumulative cell count of transduced CB cells cultured on MS5 stroma (N=3). (F) CFC colonies derived of CB cells from MS5 co-cultures (N=3). (G) CFC colonies derived from long-term culture initiating cell assay (N=3). Error bars represent standard deviation; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

duced CD34⁺ cord blood (CB) cells with control vectors (shSCR/shSCR), knockdown of *ASXL1* alone (shSCR/shASXL1), knockdown of *TP53* alone (shTP53/shSCR), or double knockdown (shTP53/shASXL1). Knockdown efficiency was determined for two independent hairpins using gene expression and western blot (Figure 1A,B). Efficacy of transduction were equal among hairpins, as shown in the *Online Supplementary Figure*. GFP/mCherry double positive CD34⁺ cells were sorted and plated into a colony-forming assay (CFC) and demonstrated reduced CFC's in the *ASXL1* transduced cells that could not be rescued by additional knockdown of *TP53* (Figure 1C,D). Upon replating, granulocytic colony formation of the *TP53/ASXL1* double knockdown was enhanced four-fold when compared to the control (hairpin #1 $P<0.01$; hairpin #2 $P<0.001$) and more than three-fold when compared to *ASXL1* and *TP53* single knockdown (hairpin #1 $P<0.05$; hairpin #2 $P<0.001$). Subsequently, transduced CD34⁺ CB cells were cultured in the context of stromal

micro-environment. Long-term stromal cultures with a MS5 stromal layer were initiated, and a comparable degree of expansion was observed in the different subgroups of transduced cells (Figure 1E). At week 1, cells taken from suspension and plated in CFC demonstrated a strong decline of CFU-GM numbers in the shASXL1 cells, which could be rescued by additional down-regulation of p53 (Figure 1F). Next, we used the long-term culture initiating cell (LTC-IC) assay as a read-out for the ability of HSPC to form colonies after an extended period in culture (Figure 1G). The data shows that loss of *ASXL1* significantly reduces the cobblestone forming potential (nine-fold difference, control vs. shASXL1, $P<0.05$) and, with additional knockdown of *TP53*, rescues the phenotype (six-fold difference, shASXL1 vs. shASXL1/shTP53, ns). Taken together, these data demonstrate that an additional knockdown of *TP53* with *ASXL1* gives advantage to HSPCs.

Following the detrimental effects of *ASXL1* knockdown alone on erythroid colony formation (Figure 1C),

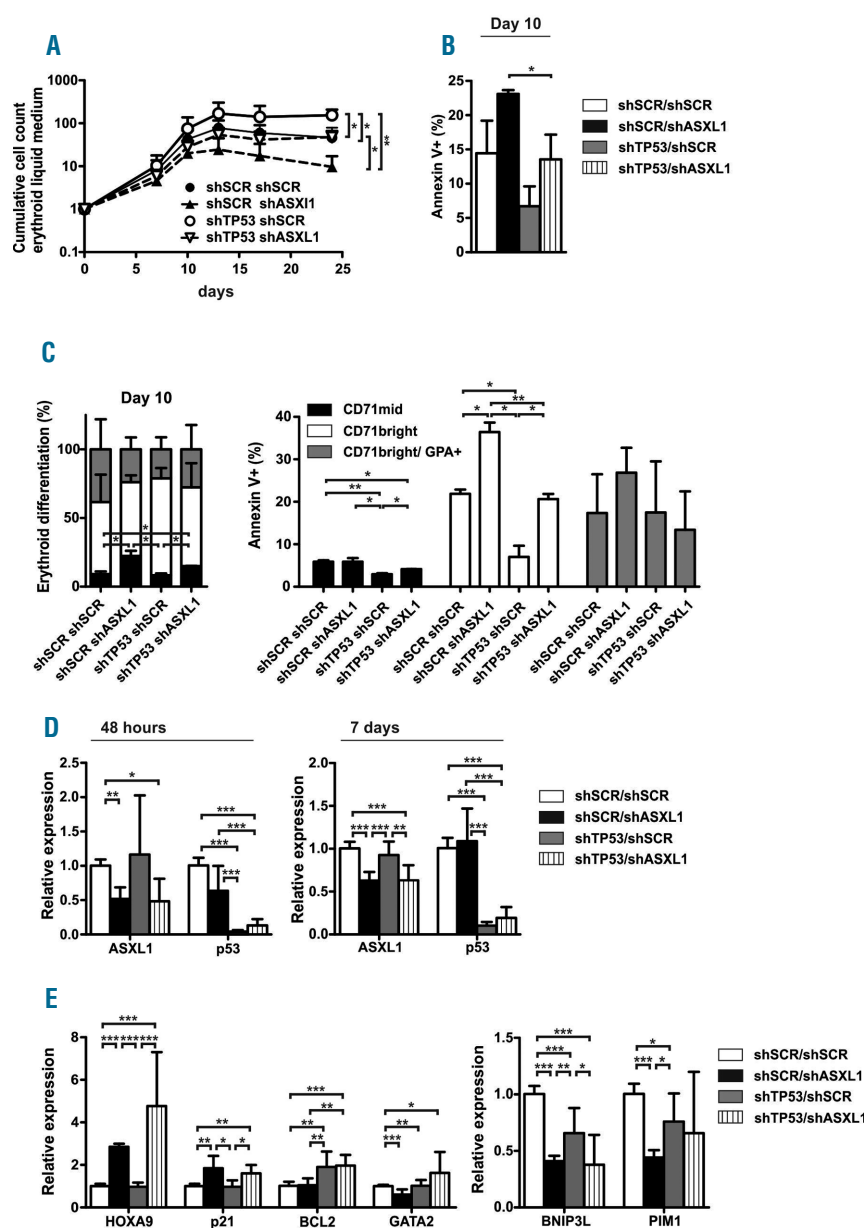


Figure 2. TP53 knockdown partially rescues shASXL1 induced apoptotic phenotype of erythroid cells. (A) Cumulative cell count of transduced CB cells cultured under erythroid permissive conditions (N=3). (B) Total percentage of Annexin V+ cells at day 10 in erythroid liquid cultures (N=3). (C) Percentage of erythroid differentiating cells at different stages and their percentage of Annexin V+ at day 10 of erythroid liquid cultures (N=3). (D) Gene expression of ASXL1 and TP53 normalized to NACA and RPS11 at 48hours (N=2) and day 7 of erythroid liquid cultures (N=4). (E) Gene expression normalized to NACA and RPS11 at day 7 of erythroid liquid cultures (N=4). Error bars represent standard deviation; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

shASXL1 and shTP53/shASXL1 CD34⁺ cells were cultured under erythroid permissive conditions. Persistent during culturing, shTP53/shASXL1 cells had a growth advantage over shASXL1 cells (Figure 2A). On day 24, cells with a double knockdown had a similar cumulative cell count to control cells and a significantly higher cell count than *ASXL1* single cells ($P<0.05$). The knockdown of *ASXL1* was accompanied with an increase in Annexin V⁺, which was rescued with additional knockdown of *TP53* (Figure 2B, day 10, shSCR/shASXL1 vs. shTP53/shASXL1, $P<0.05$).

Throughout erythroid differentiation, cells pass through several stages of maturation. Immature cells differentiate from the CD71^{mid} compartment towards the CD71^{bright}, CD71^{bright}/GPA⁺, and GPA⁺, the final erythroid stadium. At day 10, cells with double knockdown of *TP53* and *ASXL1* revealed a significantly greater percentage of CD71^{mid} cells compared to controls and shTP53 cells (Figure 2C, $P<0.05$). The apoptotic phenotype of *ASXL1* single knockdown was significantly rescued within the CD71^{mid} compartment (Day 10, shSCR/shSCR vs.

shTP53/shASXL1, $P<0.05$) and CD71^{bright} (shSCR/shASXL1 vs. shTP53/shASXL1 $P<0.01$) (Figure 2C). A stable reduction in gene expression was observed in time (Figure 2D). Further, knockdown of *ASXL1* was associated with the upregulation of *HOXA9* and with the down-regulation of *BNIP3L*, *GATA2*, and *PIM1* (Figure 2E), which could partially be rescued in double transduced cells. When looking at potential p53 downstream targets that could be involved in the rescue (Day 7), *BCL2* appeared to be significantly changed (Figure 2E). These findings suggest that the favourable phenotype of shTP53/shASXL1 is at least due to reduced apoptosis and not to alterations in the epigenetic up-make in view of the persistent elevated expression of *HOXA9*.

Previously, our lab and others reported that by using a human scaffold setting, leukemic properties can be better maintained within a human niche than a mouse niche.⁹ We therefore argued that a human microenvironment within a mouse might be optimal for cell survival and possibly malignant transformation. To this end, we transduced CD34⁺ CB cells with shTP53/shASXL1, injected

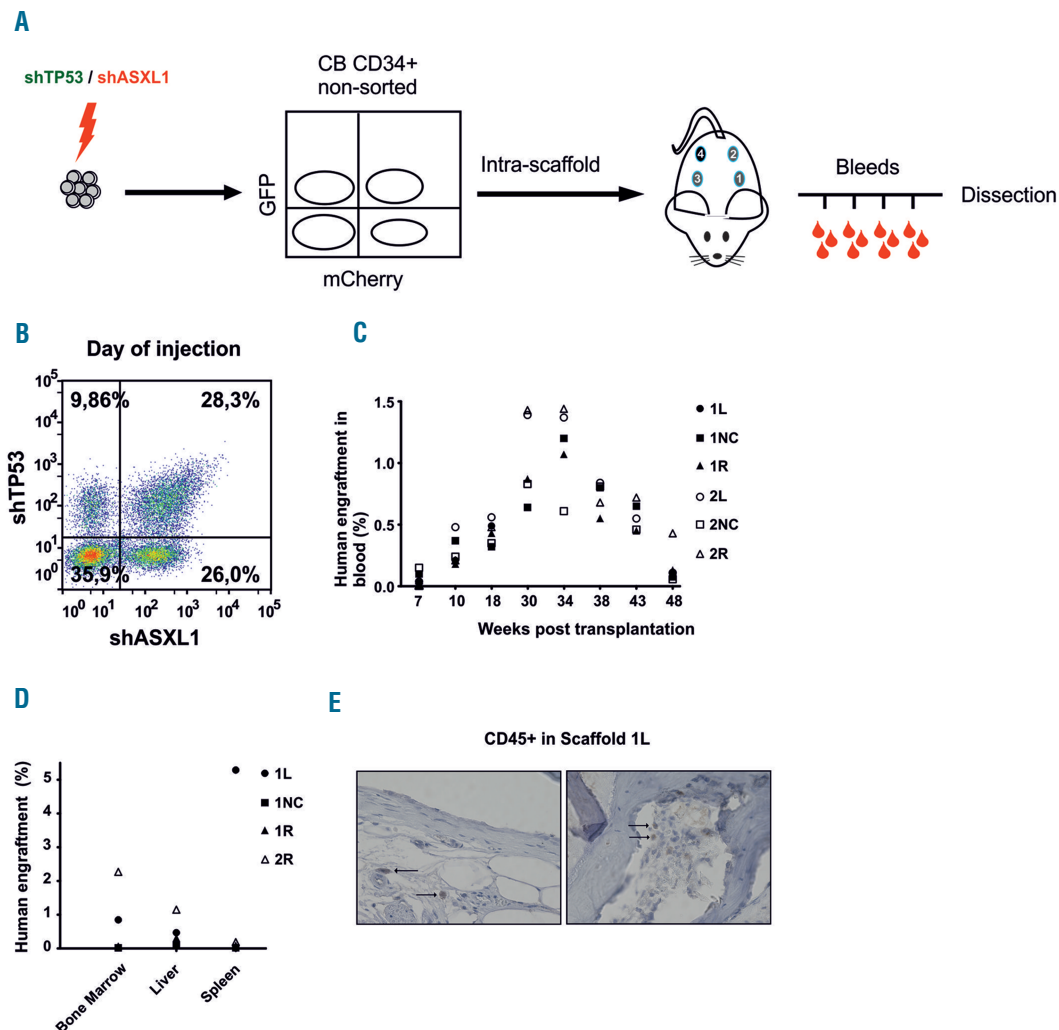


Figure 3. Humanized bone marrow-like niche in the mouse does not lead to transformation of cells with double knockdown. (A) Graphic overview of experimental set-up. Scaffold 1-3 injected with cells, scaffold 4 no cells as empty control. (B) FACS plot of transduction efficiency of shTP53 and shASXL1 at day of injection. (C) Percentage of human engraftment in the blood for six mice. Cage: 1 or 2; cut ear: L=left; R=right; NC=no-cut. (D) Percentage human engraftment in bone marrow, liver, and spleen for four mice. (E) Representative H&E staining showing development of bone and human cell engraftment (arrows).

them directly into three scaffolds per mouse ($n=6$), bled the mice in intervals and sacrificed them after 48 weeks (Figure 3A). Remaining transduced CD34⁺ CB cells were sorted for RNA to determine knockdown efficiency. Figure 3B shows the sorting gates, which were equally divided between the double knockdown and the ASXL1 single knockdown. Engraftment of human CD45⁺ cells was low throughout maturing of the mouse and declined after 34 weeks (Figure 3C). After sacrificing, bone marrow, spleen and liver were examined for human engraftment, and the *TP53* (GFP)/*ASXL1* (mCherry) percentage in the bone marrow, liver and spleen was analysed (Figure 3D). Additionally, scaffolds were embedded and examined for the presence of CD45⁺ cells but only few CD45⁺ cells could be found within the scaffolds (Figure 3E).

In this study, we demonstrated that *TP53* rescued partially the *ASXL1* phenotype by affecting the apoptotic programming without triggering malignant transformation.

Recent studies of clonal hematopoiesis in healthy donors demonstrated that *ASXL1* and *TP53* mutated cells might persist in bone marrow without overt evolution to MDS unless three or more mutations are present.^{2,10} These findings are in line with recent sequence data in MDS, particularly in high-risk MDS, that three or more mutations are frequently noticed, which might explain why in our model stem cell properties are affected but show no signs of transformation.¹¹

Mutations in the *ASXL1* gene generate a loss of function suggesting that the RNAi approach might be an appropriate way to mimic patient setting. However, recent studies have demonstrated that an overexpression of mutated *ASXL1* proteins can act within the PR-DUB pathway and globally erase and diminish H2AK119ub and H3K27me3, respectively.¹² Similar *TP53* mutations are also associated with loss of function, but recent studies have demonstrated that mutated p53 protein can also have additional functions by directly targeting chromatin modifiers and by affecting the proteasome gene transcription.^{13,14} Therefore, a combined knockdown of *ASXL1* and *TP53* may not reflect the total mutational status observed in MDS or AML. Furthermore, the order of mutation may play a crucial role in what phenotype cells present with. Ortmann *et al.* discovered that TET2 mutations preceding JAK2 V617F heavily influenced the JAK2 V617F activated transcriptional program.¹⁵ The order of mutation appeared to be of such importance that it affected the clonal evolution in patients. In this study, we simultaneously abrogated *ASXL1* and *TP53* function; in the future, an alternative approach might be used. In summary, our data demonstrate that *ASXL1*-compromised cells benefit from loss of *TP53* but do not lead to malignant transformation.

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